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Elucidation of the Enantiodiscrimination Properties of a Nonracemic Chiral Alignment Medium through Gel-based Capillary Electrochromatography: Separation of the Mefloquine Stereoisomers

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Enantiodiscrimination and enantioseparation are two highly important processes in chemistry, often performed by using NMR spectroscopy and chromatography. For a better understanding of the mechanistic details, the same system should be studied by both methods. In addition, isotropic and anisotropic NMR parameters should be obtained, the latter using alignment media so that residual dipolar couplings and chemical-shift anisotropies can be measured. Consequently, a chiral alignment medium was used for the first time in chiral gel-based capillary electrochromatography with the four stereoisomers of the antimalaria drug mefloquine as test compounds. Chromatographic data verify that enantiodiscrimination obtained with this alignment gel is caused by differences in the equilibrium constants related to associate formation. Hence, the chromatographic separation provides physicochemical data that form a basis for the understanding and optimization of alignment processes, and vice versa.

1. Introduction

Chirality plays an important role in the understanding of biological activities. Chiral drugs often show enantiospecific biological effects. Enantioseparation (ES) and enantiodiscrimination (ED) are, therefore, mandatory steps in the elucidation of enantiospecific biological functions on the molecular level. Interestingly, ED with isotropic solution NMR was first demonstrated by Pirkle,[1] who also developed enantioseparating chromatographic phases.[2] However, since these times, the two areas have evolved increasingly independently. Although NMR studies are used to elucidate structural aspects of ES,[3a] the role of enantioselective chromatography as a versatile tool in the understanding of ED obtained in NMR has been largely neglected. Since the early report of Pirkle and Pochapsky in 1986,[3b] isotropic solution-NMR studies have been used to investigate structural aspects of chromatographic enantiorecognition processes.[3c,d] For this purpose, chemical shifts and nuclear overhauser effects (NOEs) were largely used.

One recent approach for ED in NMR is the use of chiral anisotropic media.[4] In general, anisotropic achiral media permit the measurement of NMR parameters that require a partial alignment of the solute (e.g. residual dipolar couplings or chemical-shift anisotropies). Currently, a number of new alignment media that have been developed are becoming very important in the structural analysis of small-to-large molecules.[5] Cross-linked acrylamide-based co-polymers introduced in 2000[6a] have been further developed into an alignment medium compatible with a wide range of solvents including DMSO.[6b] Recently, ED was reached by introducing a chiral, negatively charged monomer: (*R*)-APhES [(2-acrylamide)-2-phenyl-ethanesulfonic acid, Scheme S1 A].[7] As expected from work with chiral ion-exchange phases,[8] ED was only reached for cationic (protonated basic) solutes (*erythro*-mefloquine, menthylamine, and strychnine). No ED was observed for the neutral compound menthol.[7]

This approach had been inspired by previous work in the field of enantioselective (chiral) liquid-phase chromatography. Enantioselective chromatographic separation is based on the difference in the retention factor for a pair of enantiomers induced by a small difference in the phase-transfer equilibrium constants, owing to the involvement of diastereomeric associations (between the chiral solute and the chiral selector) in the phase-transfer process.[8a] A special class of stationary phases developed for enantioselective liquid-phase chromatography

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(chiral ion-exchange phases) employs positively and/or negatively charged immobilized chiral selectors, which can undergo the formation of diastereomeric associates (ion pairs) with protonated or deprotonated (ionisable) chiral solutes.[8b,c]

It is now of interest to investigate whether or not the successful ED observed with a chiral alignment gel used for aniso-NMR (NMR spectroscopy under anisotropic conditions) can be further elucidated by using chromatographic studies employing the same alignment gel as a chiral separation medium in capillary electrochromatography (CEC) (Figure 1, and explanations given therein). For clarity, this approach should be clearly distinguished from techniques such as NMR-CE, capillary NMR, and chromatographic NMR spectroscopy.[9]

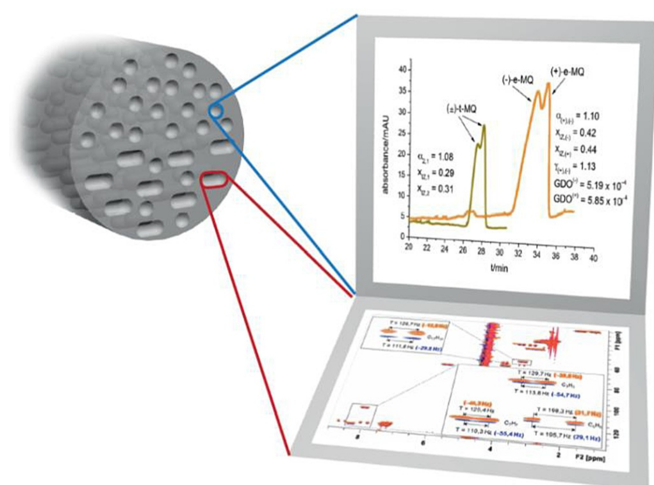

Figure 1. Left side: schematic capillary/NMR tube cross section used for isotropic gel-based capillary electrochromatography (CEC; upper part with isotropic mesh openings), and NMR under anisotropic conditions (aniso-NMR; lower part with anisotropic mesh openings). Right side: electrochromatogram obtained by CEC (upper part), and two-dimensional NMR spectrum with split signals attribtued to anisotropic alignment of the analyte (lower part).

Ideally, for thermodynamic studies, the separation medium investigated through chromatographic studies should be identical to the alignment medium used for aniso-NMR. Many alignment media (e.g. bicelles, phages), however, are incompatible with CEC instrumentation, whereas homogeneous gels are separation media in plate gel electrophoresis (PGE), in capillary gel electrophoresis (CGE), and in gel-based capillary electrochromatography (GBCEC).[10] Separation in PGE and CGE is based on differences in the migration velocity of the solute, induced by differences in the effective electrophoretic mobility of the solutes and the sieving effect. Separation in GBCEC (in analogy to chromatography), however, is based on differences in the equilibrium constant characterizing the transfer of the solute from the electrokinetic transport zone (ETZ, where the velocity of the solute is given by the electrophoretic velocity of this solute and the electroosmotic velocity) into the interaction zone (IZ, where the velocity of the solute is zero). We describe this equilibrium as a distribution process, although the highly swollen gel employed in GBCEC is a homogenous phase (in contrast to the system of two interpenetrating continuous phases characteristic for CEC with a monolith). GBCEC was introduced by Fujimoto,[10a] using charged amphiphilic polyacrylamide gels for the separation of uncharged low-molecular-weight compounds. GBCEC with hydrophilic or amphiphilic gels with embedded or immobilized chiral selectors was also successfully applied to the separation of different enantiomers.[10c–g]

Returning to previous results,[7] we selected the synthetic antimalaria drug mefloquine (Figure 2) with two stereocenters as the test solute.[11a] In recent years, the correct absolute configurations of the enantiomers of the *erythro* form, which is administered as the racemate, and of the enantiomers of the *threo* form, have been subject to debate. They were not reliably established until 2012–2013.[11b–f] In contrast to these difficulties, ES of the four stereoisomers had been successfully achieved much earlier by using capillary electrophoresis (CE) with β-cyclodextrin derivatives or other native cyclodextrins as chiral selectors,[12] and by using high-performance liquid chromatography (HPLC) either with amylose tris-3,5-dimethylphenyl carbamate-coated silica gel[13] or with a quinidine-based zwitterionic chiral stationary phase (chiral selector covalently anchored to the chemically modified silica gel).[14]

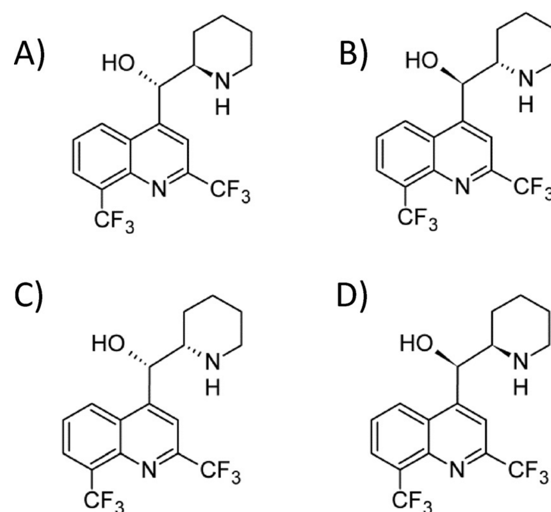

Figure 2. Structural formulae of A) (+)-*erythro*-mefloquine [(+)-e-MQ], B) (−)-*erythro*-mefloquine [(−)-e-MQ], C) (+)-*threo*-mefloquine [(+)-t-MQ], and D) (−)-*threo*-mefloquine [(−)-t-MQ].

Against this background, in the present work, we study the chromatographic separation of the four stereoisomers of mefloquine [(±)-*threo*-mefloquine (t-MQ) and (±)-*erythro*-mefloquine (e-MQ)] by using GBCEC, employing the same polymeric gel as the chiral separation medium as that already successfully taken for the ED of e-MQ through aniso-NMR. The retention data gained by using GBCEC will permit the determination of the ratio of (distribution) equilibrium constants. With this ratio, we confirm those parameters that have been calculated in the interpretation of previously reported anisotropic NMR data.[7]

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2. Results and Discussion

2.1. Chromatographic Separation

With all types of gels obtained by varying the solvent composition, a good diastereoselective and enantioselective separation of the four mefloquine stereoisomers was achieved (Figure 3). Although the peak of propranolol, taken as a marker of the hold-up time (see the Supporting Information), can be characterized to be symmetric, the peaks for the more retarded stereoisomers of mefloquine show a characteristic (right-skewed) triangular broadened shape, which can be attributed to electromigration dispersion resulting from local electric field strength inhomogeneities.

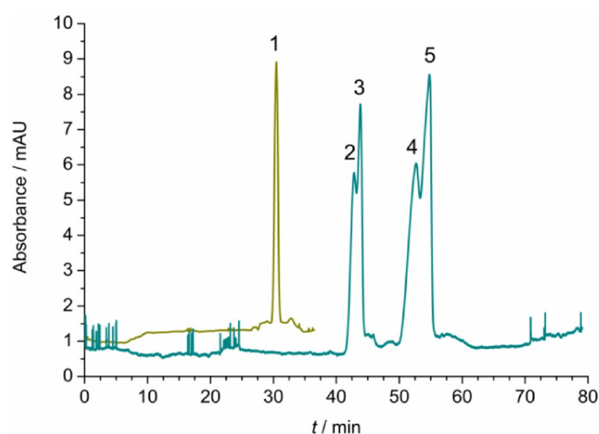

Figure 3. Separation of the four stereoisomers of mefloquine by GBCEC with alignment gel as the separation medium with superimposed scaled trace for (±)-propranolol. Peak assignment: 1) (±)-propranolol; 2, 3) (±)-*threo*-mefloquine; 4) (−)-*erythro*-mefloquine; 5) (+)-*erythro*-mefloquine; mobile phase = methanol/water (50:50, v/v) buffered with triethylamine/acetic acid, pH* = 6.66, electric conductivity = 125 μS cm^{-1}, capillary dimensions = 173 mm (102 mm) × 100 μm, photometric in-gel detection = 283 nm, electrokinetic injection = 2 kV × 2 s, separation voltage = 2.0 kV, *c* (analyte in sample) = 0.25 g L^{-1} (analyte dissolved in mobile phase).

We confirmed the suitability of propranolol as a marker of the hold-up time by application of a second independent method, which is based on the comparison of retention times obtained for the two enantiomers of e-MQ with two capillaries filled with alignment gel having different effective lengths (see the Supporting Information). It should be noted that, in GBCEC, the hold-up time can only be measured correctly if a marker is available that is not significantly retarded and has exactly the same effective electrophoretic mobility as the effective electrophoretic mobility of the analyte in the electrokinetic transport zone.

The depicted peak distortions (Figure 3) are typical in CEC for charged analytes showing a strong interaction with the oppositely charged stationary phase (ion-exchange CEC).[15] With analogously synthesized monolithic separation capillaries [employing (*R*)-APhES as negatively charged co-monomer] synthesized for comparison purposes under phase-separation conditions (see the Supporting Information), left-skewed and right-skewed peaks can be obtained, dependent on the separation conditions (Figures S1 A and S1 B).

Under the conditions selected for recording the chromatogram depicted in Figure 4 (see legend), peak broadening by electromigration dispersion is absent and a higher peak efficiency is obtained. Under these conditions, the method enabled baseline separation of the enantiomers of e-MQ (Figure 4). Spiking the sample with (+)-e-MQ HCl revealed a higher retention factor for (+)-e-MQ than for (−)-e-MQ (Figure S2). Repeatability of the data was confirmed by sequential runs (Figure S3–S5). For five repeated runs, the relative standard deviations of the observed mobilities are 2.0–2.3 % (Table S1). This corresponds to a relative confidence range of the mean of 2.5–2.9 % (*P* = 0.95, two-tailed test).

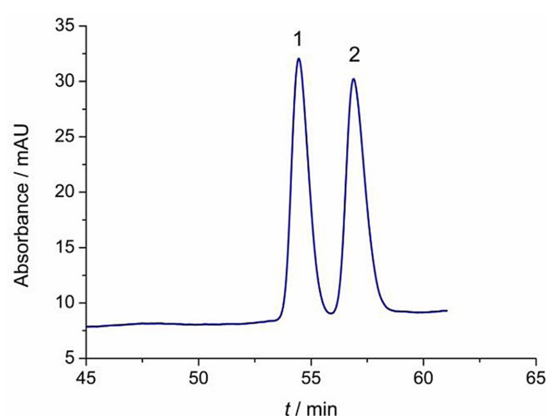

Figure 4. Separation of the enantiomers of *erythro*-mefloquine by GBCEC with alignment gel as the separation medium. Peak assignment: 1) (−)-*erythro*-mefloquine; 2) (+)-*erythro*-mefloquine; mobile phase = methanol buffered with 25 mM acetic acid and 2.66 mM triethylamine, pH* = 4.46, electric conductivity = 55 μS cm^{-1}, capillary dimensions = 234 mm (167 mm) × 100 μm, photometric in-gel detection = 283 nm, electrokinetic injection = 2.5 kV × 2 s, separation voltage = 6.1 kV, *c* (analyte in sample) = 0.25 g L^{-1} (analyte dissolved in mobile phase).

2.2. Selectivity Factor

Following two different approaches (see the Supporting Information), we estimated the chromatographic separation factor α (= ratio of the two retention factors) for t-MQ to be 1.08 and for e-MQ to be 1.10. These two factors correspond to the ratio of the two equilibrium constants related to the transfer from the electrokinetic transport zone to the interaction zone. Hence, the energetic differences $\Delta\Delta G^{\circ}$ for the involved (distribution) equilibria are 190 and 230 J mol^{-1} for t-MQ and e-MQ, respectively. Taking propranolol as a non-retarded marker with an effective electrophoretic mobility similar to that of t-MQ and e-MQ (Figure 3 and Scheme S1 B), we obtained the following retention factors: 0.41 and 0.44 for the two enantiomers of t-MQ, 0.73 for (−)-e-MQ, and 0.80 for (+)-e-MQ. A larger difference in retention factors was obtained for the separation of t-MQ and e-MQ, which corresponds to $\Delta\Delta G^{\circ} = 1.7$ kJ mol^{-1}.

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2.3. Generalized Degree of Order

Under isotropic conditions, anisotropic parameters such as dipolar couplings between two NMR-sensitive nuclei are averaged to zero.[16a] So-called alignment media lead to a low degree of orientation and re-introduce observable couplings. By using only a weak alignment, the large dipolar couplings that are in the range of kHz are scaled down to values in the range of Hz (residual dipolar couplings).[16b] With a set of experimentally determined RDCs, an alignment tensor (a matrix with five independent elements) can be calculated, which describes the degree and orientation of the alignment. In 2001, the concept of the generalized degree of order (GDO) was introduced to describe the dynamics of protein fragments.[17] GDO is a scalar quantity that is calculated from the alignment tensor (or the order tensor) through the determination of the Euclidian norm of this matrix.[16a] The GDO can be employed to characterize differences in the strength of two alignments, as it is a quantity that is only dependent on the extent of dynamic averaging, arising from both overall alignment effects and internal motional effects (the latter being neglected with rigid molecules).[16a, 17]

For (−)-e-MQ [in the gel prepared with (*R*)-APhES] $GDO^{(-)}=5.19\times10^{-4}$, which is smaller than for (+)-e-MQ [$GDO^{(+)}=5.85\times10^{-4}$; absolute difference: 0.66×10^{-4}].[7] Under the assumption that a weaker alignment correlates with a weaker interaction of the solute with the polymer chains of the gel (being identical with a less stable diastereomeric association), the determined GDOs correctly predict that (−)-e-MQ elutes first in a chromatographic separation. The significance of this result was confirmed by a duplicate determination of GDO for (−)-e-MQ.[7] $GDO_1^{(-)}=5.25\times10^{-4}$ and $GDO_2^{(-)}=5.13\times10^{-4}$, resulting in a difference of 0.12×10^{-4}. The confidence range of the arithmetic mean ($P=0.95$, one-tailed test) is 0.38×10^{-4}, which is smaller than the difference between the values for the two isomers.

With the aim to identify fragments of a protein, where motion has an effect on the accuracy of structure determination, Tolman et al.[17] defined a fragment-specific internal GDO as the ratio of the observed fragment GDO to the alignment tensor GDO. We propose here the enantioselectivity parameter β (=GDO ratio), referring to a nonracemic chiral alignment medium and a specific enantiomer pair. This parameter is quantified by determining the ratio of the (higher) GDO for Enantiomer 1 to the (smaller) GDO for Enantiomer 2 (with $\beta\geq1$). In the present case, β(e-MQ)$=GDO^{(+)}/GDO^{(-)}=1.13$. It is interesting to compare this value to the selectivity factor α ($=k^{(+)}/k^{(-)}$) obtained for (±)-e-MQ by GBCEC, which is 1.10. Both methods agree in terms of the sign [(+)>(−)] and magnitude of the quantity describing ED/ES, which should hold for cases in which the degree of alignment prevails the enantiospecific difference of the measured anisotropic parameter (here: RDC).

Based on the retention factors determined by GBCEC, it can be now concluded that the ED observed in the present case in aniso-NMR is directly related to the different degrees of transfer of the solute from the ETZ into the IZ. This degree of transfer is quantified by the molar fraction $x_{IZ}=n_{IZ}/(n_{ETZ}+n_{IZ})$. It is directly accessible from chromatographic data through $x_{IZ}=k/(1+k)$. For the different stereoisomers of mefloquine, it represents the fraction of those molecules being associated with the charged moieties of the cross-linked polymer acting as interaction sites. Although n_{total} ($=n_{ETZ}+n_{IZ}$) is kept constant, the values for x_{IZ} are 0.29 and 0.31 for the two enantiomers of t-MQ, 0.42 for (−)-e-MQ, and 0.44 for (+)-e-MQ.

3. Conclusions

Taking a chiral alignment medium as the chiral separation medium, GBCEC separates the four stereoisomers of mefloquine. This separation confirms our previous hypothesis that ED with this gel is based on the formation of localized diastereomeric associates between the charged solute and the oppositely charged moiety of the anisotropically deformed gel.[7] Consequently, ED is attributed to differences in the equilibrium constants related to associate formation. Through GBCEC, retention data are obtained that permit the calculation of the degrees of transfer of the solute from the electrokinetic transport zone into the interaction zone and the precise quantification of the Gibbs energy difference regarding the two diastereomeric associates involved in the enantioselective separation process. Hence, our approach provides physicochemical data that are important in the understanding and optimization of alignment processes.[18]

Experimental Section

The capillary pre-treatment procedure and the subsequent in situ synthesis of the polymeric gel are described in detail in the Supporting Information. Briefly, the separation capillaries are treated with a 30% (v/v) solution of 3-(trimethoxysilyl) propylmethacrylate (bind silane) in acetone to introduce vinylic anchoring groups to the inner wall of the fused silica capillary. These vinylic anchoring groups enable the covalent attachment of the synthesized gel to the inner capillary wall, which is a prerequisite to obtain homogeneously filled capillaries. Subsequently, the chiral monomer (2-acrylamide)-2-phenyl-ethanesulfonic acid [(*R*)-APhES], is co-polymerized with *N*,*N*-dimethylacrylamide (DMAA) and *N*,*N*-methylenebisacrylamide (BIS) through a free-solution radical co-polymerization (15 min at 70 °C).

The preparation procedure of the gel within the capillary is identical (with respect to concentration of monomers, reaction time, reaction temperature) to the one used for the measurement of aniso-NMR parameters with the exception of the absence or presence of a washing step (removal of non-reacted monomers), an additional drying and swelling step (in the case of aniso-NMR), and the solvent composition.[7] Although DMSO was employed for aniso-NMR, the solvent is either methanol or methanol/water [(50:50, v/v) or (25:75, v/v)] in GBCEC (after equilibration). Previous studies, however, with either methanol or DMSO as the solvent demonstrated that the type of solvent has only a minor influence on the determined aniso-NMR parameters.[19] In GBCEC, the separation gel is buffered with triethylamine/acetic acid, pH*=4.5–6.7, whereas in aniso-NMR all experiments were performed in unbuffered gels.

The influence of the electrolyte concentration on the alignment properties of a very similar achiral polyacrylamide gel [APhES re-

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placed by 2-(acrylamide)-2-methyl-propanesulfonic acid (AMPS)] was determined by Trigo-Mouriño et al.[20] According to these results, we can assume a negligible influence of the selected buffer concentration (2.7 mM) on alignment properties and retention behavior. The GBCEC apparatus employed has already been described by Wahl et al. (for details refer to the Supporting Information).[21] A scheme of the apparatus allowing in-gel detection at 283 nm is shown in Scheme S2.

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Keywords: capillary electrochromatography · enantiomers · mefloquine · NMR spectroscopy · residual dipolar coupling

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